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Review article

Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications

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Abstract

This review presents a critical analysis of covalently and ionically crosslinked chitosan hydrogels and related networks for medical or pharmaceutical applications. The structural basis of these hydrogels is discussed with reference to the specific chemical interactions, which dictate gel formation. The synthesis and chemistry of these hydrogels is discussed using specific pharmaceutical examples. Covalent crosslinking leads to formation of hydrogels with a permanent network structure, since irreversible chemical links are formed. This type of linkage allows absorption of water and/or bioactive compounds without dissolution and permits drug release by diffusion. pH-controlled drug delivery is made possible by the addition of another polymer. Ionically crosslinked hydrogels are generally considered as biocompatible and well-tolerated. Their non-permanent network is formed by reversible links. Ionically crosslinked chitosan hydrogels exhibit a higher swelling sensitivity to pH changes compared to covalently crosslinked chitosan hydrogels. This extends their potential application, since dissolution can occur in extreme acidic or basic pH conditions.

Keywords: Biomedical applications; Chitosan; Crosslinking; Hydrogel; Interaction; Review; Structure

1. Introduction

Chitosan is a copolymer of β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose. This polycationic biopolymer is generally obtained by alkaline deacetylation from chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps [1]. The main parameters influencing the characteristics of chitosan are its molecular weight (MW) and degree of deacetylation (DD), representing the proportion of deacetylated units. These parameters are determined by the conditions set during preparation. Moreover, they can be further modified. For example, the DD can be lowered by reacetylation [2] and MW can be lowered by acidic depolymerisation [3].

Chitosan is currently receiving a great deal of interest for medical and pharmaceutical applications. The main reasons for this increasing attention are certainly its interesting intrinsic properties. Indeed, chitosan is known for being biocompatible allowing its use in various medical applications such as topical ocular application [4], implantation [5] or injection [6]. Moreover, chitosan is metabolised by certain human enzymes, especially lysozyme, and is considered as biodegradable [7,8]. In addition, it has been reported that chitosan acts as a penetration enhancer by opening epithelial tight-junctions [9,10]. Due to its positive charges at physiological pH, chitosan is also bioadhesive, which increases retention at the site of application [11,12]. Chitosan also promotes wound-healing [13,14] and has bacteriostatic effects [15,16]. Finally, chitosan is very abundant, and its production is of low cost and ecologically interesting [17]. In medical and pharmaceutical applications, chitosan is used as a component in hydrogels.

This review is focused on chitosan hydrogels intended for medical or pharmaceutical applications. There are

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several possible definitions of a hydrogel; we will use the one given by Peppas [18] who defined hydrogels as macromolecular networks swollen in water or biological fluids. Examples of networks related to hydrogels that correspond to this definition will also be introduced. Due to the various possible definitions of a hydrogel, different methods of classification are possible. Based on the definition given here, hydrogels are often divided into three classes depending on the nature of their network, namely entangled networks, covalently crosslinked networks and networks formed by secondary interactions. The latter class contains all the intermediary cases situated between the two other classes representing the extremes [19]. However, with respect to chitosan hydrogels, this classification is not entirely suitable. Certainly, there are no strict borders between these classes, but there is a continuum of various gels ranging from entangled chitosan hydrogels to covalently crosslinked chitosan hydrogels. Therefore, we suggest the following modified classification for chitosan hydrogels, i.e. the separation of chemical and physical hydrogels. Chemical hydrogels are formed by irreversible covalent links, as in covalently crosslinked chitosan hydrogels. Physical hydrogels are formed by various reversible links. These can be ionic interactions as in ionically crosslinked hydrogels and polyelectrolyte complexes (PEC), or secondary interactions as in chitosan/poly(vinyl alcohol) (PVA) complexed hydrogels, grafted chitosan hydrogels and entangled hydrogels. The latter are formed by solubilisation of chitosan in an acidic aqueous medium [4,20,21], which is the simplest way to prepare a chitosan hydrogel. Entangled chitosan hydrogels will not be discussed further in this review, as they are limited by their lack of mechanical strength and their tendency to dissolve. Moreover, they do not exhibit characteristics that allow drug delivery to be efficiently controlled—such as the modification of their properties in response to changes in their physicochemical environment, such as pH or temperature.

The present review is exclusively concerned with chitosan hydrogels formed by the addition of a crosslinker, namely covalently or ionically crosslinked hydrogels. A second review, entitled 'Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications' will discuss hydrogels formed by direct interaction between polymeric chains, without the addition of crosslinkers. They can be formed by complexation with another polymer, generally ionic, or by aggregation after chitosan grafting [22]. In crosslinked hydrogels, polymeric chains are interconnected by crosslinkers, leading to the formation of a 3D network (Fig. 1). Crosslinkers are molecules of MW much smaller than the MW of the chains between two consecutive crosslinks [23]. The properties of crosslinked hydrogels depend mainly on their crosslinking density, namely the ratio of moles of crosslinking agent to the moles of polymer repeating units [23]. Moreover, a critical number of crosslinks per chain is required to allow the formation of a network, such as that of

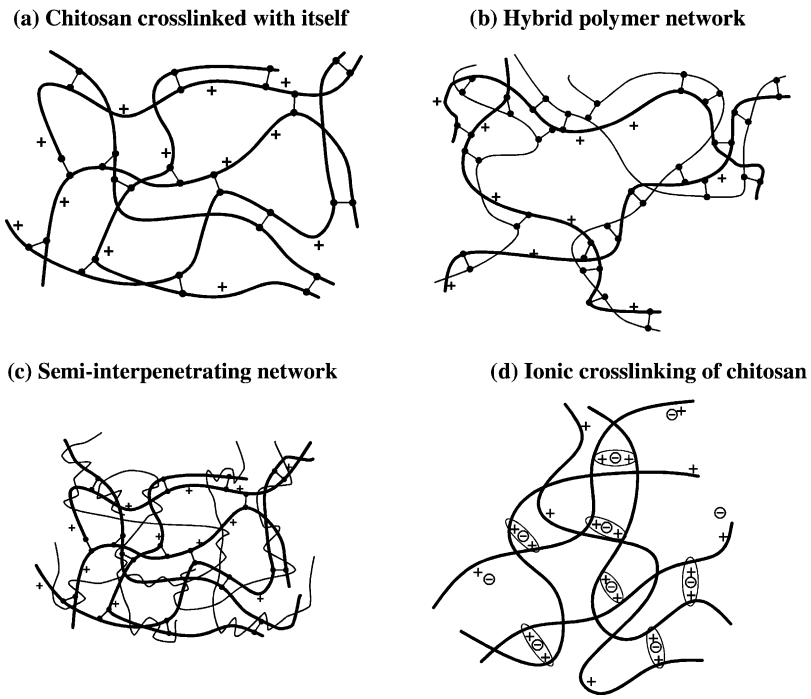

Fig. 1. Structure of chitosan hydrogels formed by (a) chitosan crosslinked with itself; (b) hybrid polymer network; (c) semi-interpenetrating network; (d) ionic crosslinking; ●—●, covalent crosslinker; +, positive charge of chitosan; ▬, chitosan; —, additional polymer; ⊖, charged ionic crosslinker; ⬭, ionic interaction.

a hydrogel [24]. Depending on the nature of the crosslinker, the main interactions forming the network are covalent or ionic bonds. The structures and interactions forming the covalently and ionically crosslinked hydrogels will be presented, their principles of formation and properties will be considered and examples of medical or pharmaceutical applications will be given. Their potential biocompatibility will be discussed, although some examples will refer to systems that are still in development, while others have already been tested in animals. To date, only one hydrogel presented in this review has been tested in humans. Consequently, their potential biocompatibility will sometimes be evaluated based on the intrinsic biocompatibility of their components.

2. Covalently crosslinked chitosan hydrogels

2.1. Structure and interactions

Hydrogels based on covalently crosslinked chitosan can be divided into three types with respect to their structure: chitosan crosslinked with itself (Fig. 1a), hybrid polymer networks (HPN) (Fig. 1b) and semi- or full-interpenetrating polymer networks (IPN) (Fig. 1c). The simplest structure presented here is chitosan crosslinked with itself. As represented in Fig. 1a, crosslinking involves two structural units that may or may not belong to the same chitosan polymeric chain [25]. The final structure of such a hydrogel could be considered as a crosslinked gel network dissolved in a second entangled network formed by chitosan chains of restricted mobility [26]. In hydrogels formed by a HPN, the crosslinking reaction occurs between a structural unit of a chitosan chain and a structural unit of a polymeric chain of another type (Fig. 1b), even if crosslinking of two structural units of the same type and/or belonging to the same polymeric chain cannot be excluded. Finally, semi- or full-IPNs contain a non-reacting polymer added to the chitosan solution before crosslinking. This leads to the formation of a crosslinked chitosan network in which the non-reacting polymer is entrapped (semi-IPN). It is also possible to further crosslink this additional polymer in order to have two entangled crosslinked networks forming a full-IPN, whose microstructure and properties can be quite different from its corresponding semi-IPN [27].

In each of the three types of structures, covalent bonds are the main interactions that form the networks, but other interactions cannot be excluded. Indeed, secondary interactions, such as hydrogen bridges and hydrophobic interactions, occur between acetylated units of chitosan and lead to a more solid-like gel if the DD is low enough [28], but as crosslinking density increases, covalent bonds tend to become predominant. When another polymer is included, additional secondary interactions between this polymer and chitosan arise and participate in the formation of the hydrogel. For example, strong intermolecular hydrogen bonds are formed in the case of HPN with gelatine [29] or in semi-IPN with polyether [30,31], silk fibroin [32] or PEO [33].

Finally, it should be noted that the crosslinking reaction can induce a conformational change of chitosan, as observed by NMR with 1,1,3,3-tetramethoxypropane [34] and glutaraldehyde [25]. However, the influence of this change on the properties of hydrogels is not yet clearly understood and requires further investigation.

2.2. Principles of formation

Preparation of a hydrogel containing a covalently crosslinked chitosan minimally requires chitosan and a crosslinker in an appropriate solvent, usually water. Other components can be added, such as additional polymers to form a HPN or a semi- or full-IPN. Auxiliary molecules can also be used to catalyse or initiate reactions during the preparation of the network.

Crosslinkers are molecules with at least two reactive functional groups that allow the formation of bridges between polymeric chains [22]. To date, the most common crosslinkers used with chitosan are dialdehydes such as glyoxal [33,35] and in particular glutaraldehyde [36–38]. Their reaction with chitosan is well-documented; the aldehyde groups form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylenic bonds [25,39] via a Schiff reaction. However, links with hydroxyl groups of chitosan cannot be excluded [40]. Dialdehydes allow direct reaction in aqueous media, under mild conditions and without the addition of auxiliary molecules such as reducers [26,33,41,42], which is advantageous with respect to biocompatibility. However, the main drawback of dialdehydes such as glutaraldehyde is that they are generally considered to be toxic [43,44]. For example, glutaraldehyde is known to be neurotoxic, its fate in the human body is not fully understood [45] and glyoxal is known to be mutagenic [46]. Therefore, even if hydrogels are purified before administration, the presence of free unreacted dialdehydes in hydrogels could not be completely excluded and may induce toxic effects. Other covalent crosslinkers for chitosan have been investigated as alternatives. Besides dialdehydes, crosslinkers such as diethyl squarate [47], oxalic acid [48] or genipin [49] can exhibit direct crosslinking mechanisms, although they remain incompletely elucidated. However, there is a lack of data regarding the biocompatibility of diethyl squarate, while oxalic acid [48] has shown in vitro toxicity in rats [50]. The use of genipin is an interesting alternative to dialdehydes. It is a naturally occurring material, which is commonly used in herbal medicine and as a food dye [51]. The biocompatibility of genipin in humans has not been assessed yet, but it is not cytotoxic in vitro [52] and has been shown to be biocompatible after injection in rats [51]. Another approach is the formation of covalently linked networks, close to HPN, by use of

functionalised, water soluble, biocompatible polymers. Due to their high MW, these functionalised polymers do not correspond to the definition of crosslinkers used here. However, as they were specially developed to crosslink chitosan and as the properties and applications of the hydrogels they form with chitosan are similar to those of covalently crosslinked chitosan hydrogels, they are discussed here. Such functionalised biopolymers can be poly(ethylene glycol) (PEG) diacrylate [53], oxidised β-cyclodextrin [54], telechelic-PVA [54] or dialdehydes derived from PEG [55] or scleroglucan [56]. However, even if these products are known to be biocompatible before functionalisation, data concerning the biocompatibility of their functionalised derivatives are lacking. Consequently, further investigation into their role as crosslinkers is not warranted unless their use in humans is demonstrated to be risk-free.

As the HPN and IPN hydrogels presented below usually require the addition of dialdehydes as crosslinkers, their substitution by more biocompatible compounds should also be studied. The preparation of a HPN requires the use of additional polymers that bear reactive groups, able to undergo crosslinking with chitosan. For example, these can be gelatine, collagen or a silylating agent, which all bear amine groups that are crosslinked via glutaraldehyde with the amine groups of chitosan [29,57]. For the preparation of a semi-IPN, polymers without functional groups that are able to react with the crosslinker are used. Examples are polyether [58–60], poly(vinyl pyrrolidone) (PVP) [61], silk fibroin [32], PEO [33], poly(*N*-isopropylacrylamide) [42] and PEG [60]. The latter two polymers can also serve for the preparation of a full-IPN [27,60]. The incorporation of an additional polymer allows the preparation of pH-sensitive swelling systems [31–33,62–64], a characteristic that depends on the hydrophilicity of the added polymer (see swelling and drug release section). Therefore, one should pay particular attention to its choice.

Direct crosslinking in aqueous media is of course desirable, but the addition of potentially toxic auxiliary molecules is often required to initiate or catalyse polymerisation or crosslinking. For example, this is the case with the use of 2,2-dimethoxy-2-phenyl acetophenone to initiate copolymerisation of PEG during full-IPN formation [60], or with sodium cyanoborohydride, the most common grafting catalyst [22], used to promote the crosslinking reaction with 1,1,3,3-tetramethoxypropane [34], functionalised PVA [54], functionalised scleroglucan [56] or functionalised PEG [55]. These auxiliary molecules are found in traces in the final product. Since their fate in the human body is not clearly known, their use in medical and pharmaceutical applications in humans may be limited.

The formation of a full-IPN requires crosslinking of the additional polymer. This can be performed by UV irradiation to polymerise and crosslink PEG macromers [60] or by the addition of a second crosslinker, such as methylene bis-acrylamide to crosslink poly(*N*-isopropylacrylamide) networks [27]. However, the addition of a second crosslinker certainly decreases the biocompatibility.

Covalent crosslinking can also be performed after polyelectrolyte complexation with chondroitin sulfate [65], collagen [66], poly(acrylic acid) (PAA) [67] or xylan [68]. In addition, chitosan/PVA complexes [69,70] and grafted chitosan networks of poly(*N*-isopropylacrylamide)- [71], fructose- [72] or *N*,*O*-carboxymethyl–chitosan [73] can be crosslinked. In these cases, crosslinking is added in order to reinforce the complexed network and to avoid dissolution during swelling [22].

It is important to characterise the conditions of the crosslinking reaction, since they determine and allow the modulation of the crosslinking density, which is the main parameter influencing interesting properties of hydrogels such as drug release and mechanical strength. Covalent crosslinking, and therefore the crosslinking density, is influenced by various parameters, but mainly dominated by the concentration of covalent crosslinker, such as glutaraldehyde [26,36,74]. It is favoured when chitosan MW [74] and temperature increase [74,75]. Moreover, since crosslinking requires mainly deacetylated reactive units, a high DD of chitosan is favourable [28]. Obviously, crosslinking reactions are also influenced by their duration [75]. As the main parameters influencing crosslinking density have been identified, the possibilities of monitoring reaction during hydrogel formation should now be investigated to facilitate the development of tailor-made hydrogels.

2.3. Properties and medical applications

Covalent crosslinking leads to the formation of a permanent network allowing the free diffusion of water and enhancing the mechanical properties of the gel. As a result of these interesting characteristics, covalently crosslinked chitosan hydrogels have two main applications, namely as drug delivery systems allowing release of bioactive materials by diffusion and as permanent networks used, for example, as scaffolds in cell culture. It should be noted that the biocompatibility of the following examples has not yet been assessed and that due to traces of potentially toxic free auxiliary molecules or crosslinkers, the administration of such systems in humans may be problematic.

2.3.1. Swelling and drug release

Whatever the type of structure, networks containing covalently crosslinked chitosan are considered as porous [29,55,61,76]. This term is used to describe networks containing free water that can diffuse through the hydrogel. Due to these pores, covalently crosslinked chitosan hydrogels can be used as drug delivery systems from which drugs are released by diffusion [77]. Indeed, as a result of the increase or decrease of the swelling ratio, we can expect the mesh size of the network to also increase or decrease

considerably. The swelling ratio change of chitosans translates into a change in the mesh size of the gel, which modulates drug release.

In order to calculate the mesh size from the equilibrium swelling data (determined as explained below), the Peppas–Merrill equation is used to determine the number average molecular weight between crosslinks, $\bar{M}_c$:

$$\frac{1}{\bar{M}_c} = \frac{2}{\bar{M}_n} - \frac{(\bar{v}/V_1)\left[\ln(1-\upsilon_{2,s}) + \chi_1 \upsilon_{2,s}^2\right]}{\upsilon_{2,s}\left[\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{1/3} - \frac{1}{2}\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)\right]} \quad (1)$$

where $\bar{M}_n$ is the number average molecular weight of the uncross-linked polymer, $\bar{v}$ is the specific volume of the polymer, V_1 is the molar volume of the swelling medium (= 18 cm^3/mol for water), $\upsilon_{2,r}$ is the polymer volume fraction after crosslinking but prior to swelling (the relaxed polymer volume fraction), $\upsilon_{2,s}$ is the polymer volume fraction after equilibrium swelling (the swollen polymer volume fraction), and χ_1 is the Flory polymer–solvent interaction parameter. The values of $\upsilon_{2,r}$ and $\upsilon_{2,s}$ are determined by using the following relationships:

$$\upsilon_{2,r} = \frac{V_d}{V_r} \quad (2)$$

$$\upsilon_{2,s} = \frac{V_d}{V_s} \quad (3)$$

From the molecular weight between crosslinks, the number of links between two crosslinks, n, is calculated as

$$n = \frac{2\bar{M}_c}{M_r} \quad (4)$$

where M_r is the average molecular weight of the repeating unit. The value of the root mean squared end-to-end distance of the polymer chain in the freely jointed state is calculated by using Eq. (8):

$$\left(\bar{r}^2\right)^{1/2} = \ell\sqrt{n} \quad (5)$$

where ℓ is the carbon–carbon bond length. The root mean squared end-to-end distance of the polymer chain in the unperturbed state was calculated as

$$\left(\bar{r}_0^2\right)^{1/2} = \sqrt{C_n}\left(\bar{r}^2\right)^{1/2} \quad (6)$$

where C_n is the Flory characteristic ratio or rigidity factor of the polymer. Finally the mesh size of the polymer network, ξ, was determined from Eq. (7):

$$\xi = \upsilon_{2,s}^{-1/3}(\bar{r}_0^2)^{1/2} \quad (7)$$

Diffusion depends mainly on the crosslinking density, which is determined by the reaction parameters, therefore the latter can be used to monitor drug release [38,78]. The polymeric chains form a network containing many pores filled with small molecules such as water, which can be free [41] or bound to the hydrophilic groups of the network [79]. Lightly crosslinked systems form super adsorbing hydrogels in which crosslinking gives rise to a continuum of free water [79]. As the crosslinking density increases, water content, swelling capacity [80] and the mesh size of the network decrease. Indeed, increasing the amount of crosslinker decreases the ability of chitosan to form hydrogen bonds with water molecules [36]. Moreover, the higher the crosslinking density, the lower the swelling ability of chitosan hydrogels due to the slower relaxation time of the polymeric chains, which results in a decreased drug-release rate [74]. However, regardless of the crosslinking density, free molecules and free water can always move within the structure through pores and be released out of the gel [81].

The analysis of swelling ratios and equilibrium or dynamic swelling behaviour also allows one to gain insight into the drug release behaviour from the network. This can be determined on dried hydrogels disks weighed in air and in *n*-heptane and then placed in phosphate–citrate buffer solutions of pH values between 2.2 and 8.0 at 37 °C. The ionic strength of each buffer solution is adjusted to 0.5 M. After swelling, the samples are taken out of the buffer solutions, blotted to remove surface water and weighed in air and in *n*-heptane.

The weight swelling ratio, q, and the volume swelling ratio, Q, are calculated using Eqs. (8) and (9):

$$q = \frac{W_s}{W_d} \quad (8)$$

Here, W_s is the weight of the swollen hydrogel and W_d is the weight of the initially dry hydrogel. Also,

$$Q = \frac{V_s}{V_d} \quad (9)$$

where V_s is the volume of the swollen hydrogel and V_d is the volume of the initially dry hydrogel. The volume of the hydrogel in the swollen or dry state is obtained by determining its weight in air and in *n*-heptane, a non-solvent for the polymer, and calculated using the buoyancy principle with Eq. (10):

$$V = \frac{W_a - W_h}{\rho_h} \quad (10)$$

Here, V is the volume of the polymer, W_a is the weight of the polymer in air, W_h is the weight of the polymer in *n*-heptane, and ρ_h is the density of *n*-heptane (= 0.684 g/cm^3). To examine the equilibrium swelling behaviour, dried hydrogel disks were weighed and placed in the buffer solutions. Equilibrium swelling can be determined when the weight of the swollen hydrogels reaches constant value (±1%). To determine the dynamic swelling behaviour, dried hydrogel disks are weighed and placed in buffer solutions. The disks are taken out of the buffer, blotted to remove surface water and weighed at specified time intervals.

Oscillatory swelling experiments can be performed to investigate the reversibility of the swelling/deswelling

process of the polymer networks with respect to the environmental pH change. Dried hydrogel disks are swollen in a buffer solution of pH 7.0, then placed in a buffer solution of pH 2.2, and returned to a buffer solution of pH 7.0, and finally collapsed in a buffer solution of pH 2.2 again. At each step, the weights of the hydrogel disks are measured at specified time intervals. In these experiments, the pH values of the two solutions are chosen so that one solution is above the transition point of the gel, while the other solution is below the transition.

Diffusion is also influenced by the nature of the crosslinker. The chemical nature and length of the bridging units of the crosslinker influence porosity, water uptake and swelling [54,82]. For example, 1,1,3,3-tetramethoxypropane enhances the hydrophilicity of the network [34], PEG-diacrylate improves swelling [53] and depending on the length of PEG-dialdehyde, the hydrophilicity of the network and its swelling are modified [55]. Swelling and diffusion are also influenced by the hydrophilicity of the additional polymer. Indeed, the incorporation of a hydrophobic water-repelling polymer, such as polyether [31], decreases swelling volume and kinetics. This increase in hydrophobicity decreases the proportion of water bound to the polymeric matrix and increases the volume of free water and its mobility, as with the incorporation of poly(oxypropylene glycol) containing hydrophobic moieties [59]. In contrast, the addition of a hydrophilic polymer such as silk fibroin [32], poly(ethylene oxide) (PEO) [33], PVP [63] or PAA [64] increases water uptake and swelling. Consequently, in addition to crosslinking density, the physicochemical nature of the crosslinker and that of any additional polymer allow modulation of drug release and extend the range of potential applications of such hydrogels.

In hydrogels formed by chitosan crosslinked with itself, release is mostly controlled by the crosslinking density; consequently, the higher the crosslinking density, the lower the release rate [38,77,78,83]. However, other system parameters, such as drug concentration [78] often play a major role. To our knowledge, there are no examples of hydrogels formed by chitosan crosslinked with itself that exhibit pH-sensitive swelling. Indeed, the numerous interchain interactions formed by crosslinking inhibit swelling, since most of the amino groups of chitosan must have reacted with the crosslinker. Such systems do not present a release profile that can be further modulated after administration, for example, drug release cannot be targeted in the gastro-intestinal tract, which limits their range of application. The incorporation of an additional polymer, whose hydrophilicity is different from chitosan, allows pH- and ion-sensitive swelling in acidic conditions [84]. The additional polymer should perturb covalent crosslinking between chitosan chains, hence, decreasing crosslinking density and making available more protonable amino groups. This pH-sensitive swelling in an acidic environment allows the preparation of controlled drug delivery systems, the release from which is modulated by the crosslinking density and the pH of the medium. The mechanism of pH-sensitive swelling involves protonation of the amino groups of chitosan when the pH decreases. This protonation leads to chain repulsion, diffusion of proton and counter-ions together with water inside the gel and dissociation of secondary interactions [84] allowing swelling (Fig. 2). This dissociation, together with increased hydrophilicity, can explain the higher swelling degree of a semi-IPN hydrogel, containing a hydrophilic polymer. Such systems present a higher versatility than hydrogels formed by chitosan crosslinked with itself, but their higher swelling degree can lead to dissolution of the gel, as in the case of a semi-IPN containing a polyether [85]. To avoid dissolution, swelling can be reduced by an increase of the crosslinking density and/or by changing the nature of the crosslinker. For example, hydroxypropylcellulose and crosslinked chitosan semi-IPN are less soluble in water when crosslinked with

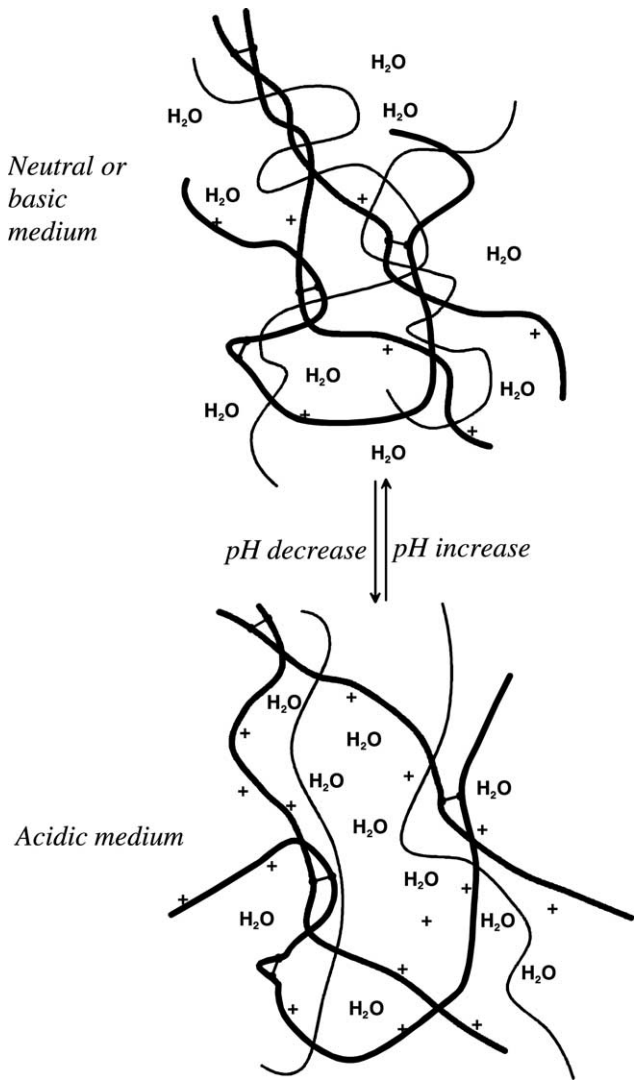

Fig. 2. pH-sensitive swelling of a semi-interpenetrating polymer network containing a crosslinked chitosan; ●—●, covalent crosslinker; +, positive charge of chitosan; ▬, chitosan; ——, additional polymer.

Table 1
Systems formed by crosslinked chitosan

Crosslinker		Controlled release systems	Biomedical applications
Dialdehyde	Glutaraldehyde	Membranes for controlled delivery of riboflavin [36].	Scaffold for hepatocyte attachment [97].
		Transdermal delivery of oxprenolol HCl [78] or propanolol HCl [88].	Scaffold for the immobilisation of enzymes producing dextranase [126] or of tyrosinase for the production of L-DOPA [127].
		Fibres for 5-fluorouracil release [38].	Gel particles for sorption of heavy metals [94].
		Freeze-dried microspheres for release of goserelin [125].	Film for the fabrication of an amperometric glucose biosensor [128].
			Conjugate for oral administration of oxalate-oxidase [92].
			Hydrogel for immobilisation of enzymes in food processing industry [105].
			Biocatalytic hydrogels conjugated with laccase [100].
			Water resistant bioadhesive [37].
			Material supporting nerve repair [129].
			Oral phosphate binder [130].
	PEG dialdehyde diethyl acetal	n.r.	Biocompatible and biodegradable wound dressing material [55].
	Formaldehyde	Matrices for sustained release of rifampicin [83].	n.r.
Various	Genipin	Injectable microspheres [51].	Biocompatible and biodegradable materials for use as implants, blood substitutes or wound dressing material [131].
		Gel beads for the controlled release of indomethacin [89].	
	Oxalic acid	Transdermal drug delivery system [132].	n.r.

n.r., no reference available.

glutaraldehyde rather than glyoxal [86]. Moreover, incorporation of an additional polymer can also induce important but non-pH-dependent swelling, e.g. polyacrylamide [76], or temperature-sensitive swelling, e.g. poly(*N*-isopropylacrylamide) [87]. However, the potential applications of these systems still need to be further investigated.

2.3.2. Examples of drug delivery systems

Systems formed by chitosan crosslinked with itself are used as drug delivery systems due to the possibility of drug diffusion. A variety of such networks related to hydrogels are listed in Table 1. Examples are transdermal drug delivery systems for the delivery of oxprenolol HCl [78] or propanolol HCl [88], fibres for the release of 5-fluorouracil [38] or gel beads for the controlled release of indomethacin [89]. It should be noted that the use of genipin allows the preparation of injectable or implantable hydrogels that exhibit in vivo biocompatibility in rats [51,90]. To our knowledge, this is the only hydrogel presented here that exhibits such characteristics.

There are only a few examples of HPN drug delivery systems (Table 2). On the other hand, semi- or full-IPN allow the preparation of various systems (Table 3) that can, for example, be employed for the administration of antibiotics, such as amoxicillin [35,61,76]. Moreover, in the treatment of gastric ulcers, chitosan combines its antiulcer properties with the activity of an antacid drug, such as cimetidine [85]. Finally, the addition of PEG allows the formation of an oral sustained delivery system with high drug-loading capacity [91]. Note that some hydrogels given

Table 2
Systems based on hybrid polymer networks containing chitosan and glutaraldehyde as crosslinker

Additional polymer	Controlled release system	Biomedical applications
Gelatine	Hydrogels for the controlled release of levamisole, chloramphenicol or cimetidine [39].	Biocompatible and biodegradable materials for use as implants, blood substitutes or wound dressing material [133].
		Matrix containing hydroxyapatite as bone-replacing composite [29].
		Material supporting nerve repair [129].
Collagen	n.r.	Scaffold for culture of human epidermoid carcinoma cells and test of anticancer drugs [102].
Silylating agent	n.r.	Hydrogels for enzyme immobilisation or copper adsorption [57].

n.r., no reference available.

Table 3
Semi- or full-interpenetrating polymer networks containing crosslinked chitosan

Crosslinker		Additional polymer	Controlled release system	Biomedical applications
Dialdehydes	Glutaraldehyde	Silk fibroin	n.r.	Artificial muscle converting chemical energy into mechanical energy [32].
		Poly(*N*-isopropylacrylamide)	pH- and temperature-sensitive gel [87].	n.r.
		Polyether	Hydrogel for the controlled delivery of cimetidine [85] or chlorhexidini acetas [84]. Gel beads with high drug-loading capacity for oral delivery [91].	Anticoagulant hydrogel containing heparin [134].
		Polyvinyl pyrrolidone	Freeze-dried hydrogel for the controlled release of amoxicillin [61].	Hydrogel supporting growth of epithelial cells and inhibiting fibroblast growth for wound healing [104]. Semipermeable hydrogel membranes for islet immunoisolation and transplantation [103,123].
		Poly(vinyl alcohol)	Membrane for the sustained release of vitamin B-12 [135]. Membrane for the pH-controlled release of insulin [136].	Membrane for nanofiltration [70].
	Glyoxal	Polyethylene oxide	Hydrogels for site-specific antibiotics delivery in the stomach [35].	n.r.
Various	*N*,*N*'-Methylene bisacrylamide	Polyacrylamide	Biocompatible hydrogel for controlled delivery of amoxicillin [76].	n.r.

n.r., no reference available.

as examples in the following tables have already been tested *in vitro* for drug delivery, while others only represent potential drug delivery systems.

2.3.3. Enhanced mechanical strength and cell or enzyme immobilisation

The enhanced mechanical strength induced by covalent crosslinking and secondary interactions allows various uses that are not possible with systems containing only entangled chitosan. This enhanced mechanical strength together with the good absorption properties of crosslinked chitosan networks [92] are mainly interesting for cell or enzyme immobilisation. This good absorption property is also interesting in ion sorption, for example, for the removal of heavy metals from water [93–95]. For this purpose, the crosslinker can also play an active role in the network, e.g. glutaraldehyde that participates in heavy metal ion sorption via free aldehyde groups [96].

As listed in Table 1, chitosan crosslinked with itself by glutaraldehyde forms hydrogels that can be used as scaffolds in hepatocyte attachment [97]. They can also serve to immobilise enzymes. The activity of enzymes can be further improved by substitution of glutaraldehyde with alternative crosslinkers, such as tris(hydroxymethyl)phosphine [98] or by crosslinking after the addition of the enzymes allowing improved repartition [99]. Moreover, it is possible to conjugate enzymes, such as laccase, with chitosan, which improves the stability and activity of enzymes at extreme acidic or basic pH [100].

Since collagen is found in skin and bone matrix, a HPN containing chitosan and collagen presents biological and mechanical benefits for use as a cellular scaffold [101]. For example, crosslinking chitosan and collagen allows the preparation of a scaffold for human epidermoid carcinoma cell cultures [102]. Moreover, addition of hydroxyapatite forms a bone-replacing porous material [29]. Additional examples are given in Table 2.

Formation of a semi-IPN allows modification of the physico–mechanical properties of a hydrogel. For example, addition of a polyether [30], such as a PEO [33] increases the flexibility of the gel, while the viscoelastic nature of PVP enhances the mechanical strength [63]. These improved physico–mechanical properties are useful in various applications, as for the preparation of nanofiltration membranes for ionic or organic solutes [70], the formation of cell culture media for islet immunoisolation [103] or for wound-healing management by growth modulation of fibroblasts [104] (Table 3). Moreover, the semi-IPN structure can be more favourable than chitosan crosslinked with itself for cultivating cells. For example, addition of gelatine after crosslinking improves immobilisation yields and mechanical properties and decreases enzyme release [105]. Finally, the addition of poly(*N*-isopropylacrylamide) in order to prepare a semi- or full-IPN, modifies

the transparency of the system, in a temperature-dependent manner [27,42]. However, no application is proposed yet for these hydrogels.

3. Ionically crosslinked chitosan hydrogels

Most of the crosslinkers used to perform covalent crosslinking may induce toxicity if found in free traces before administration. A method to overcome this problem and to avoid a purification and verification step before administration is to prepare hydrogels by reversible ionic crosslinking. Chitosan is a polycationic polymer, well known for its chelating properties [93]. Therefore, reactions with negatively charged components, either ions or molecules, can lead to the formation of a network through ionic bridges between polymeric chains, whose presence can be demonstrated by IR spectra [106], turbidimetric titration [107] or viscosimetry [24]. Since the nature of these interactions is the same as in PEC, it is difficult to classify separately these two types of network. Indeed, crosslinking is usually considered as a bridge of MW much smaller than the MW of the chains between two consecutive crosslinks [23] and the MW of a small polymer forming a PEC and of a large molecule allowing ionic crosslinking could converge and become very close. However, the classification of the examples presented in this review is simpler. In ionic crosslinking, the entities reacting with chitosan are ions or ionic molecules with a well-defined MW. In contrast, in polyelectrolyte complexation, the entities reacting with chitosan are polymers with a broad MW distribution [22].

3.1. Structure and interactions

Ionically crosslinked chitosan networks can be divided into two groups depending on the type of crosslinker used, either anions or anionic molecules. However, most of their characteristics and properties are identical (see properties and uses). Their structures, as represented in Fig. 1d are very similar. A network is formed in the presence of negatively charged entities, which form bridges between the positively charged chitosan polymeric chains.

Ionic interactions between the negative charges of the crosslinker and positively charged groups of chitosan are the main interactions inside the network. Their nature depends on the type of crosslinker. Metallic ions, which are commonly used, induce the formation of coordinate-covalent bonds between positively charged ammonium groups of chitosan [24]. This type of bond is a stronger link than the electrostatic interactions formed by the anionic molecules used as crosslinker. In addition to the positively charged ammonium groups of chitosan, other groups along the chitosan chains, such as hydroxyl groups can also react with the ionic crosslinker [108]. Moreover, additional interactions can occur inside the network, such as hydrophobic interactions favoured by a decrease of the DD of chitosan [109] or interchain hydrogen bonds due to the reduced electrostatic repulsion after neutralisation of chitosan by the crosslinker [24,107,109]. Furthermore, simultaneous formation of a PEC with an additional polymer, such as chondroitin sulphate, can also occur [110].

3.2. Principles of formation

In order to prepare a polymeric network containing ionically crosslinked chitosan, one needs at least a charged ionic crosslinker and chitosan dispersed in a solvent, commonly water. Moreover, an additional polymer can be incorporated. As covalent crosslinking requires multifunctional molecules as crosslinkers, ionic crosslinking requires multivalent counter-ions as crosslinkers to form bridges between polymeric chains. As chitosan is a polycation, anions or anionic molecules are generally required, but some cases are known where a negatively charged chitosan derivative is ionically crosslinked by cations, such as Fe(III) [108].

Metallic anions, as Mo(VI) [111] or Pt(II) [24], are commonly used as ionic crosslinkers. Among anionic molecules, phosphate-bearing groups, such as β-glycerophosphate [112] and particularly tripolyphosphate [106,113] are commonly used. One should note here that β-glycerophosphate does not seem to induce ionic crosslinking [114]. However, the nature of its interaction with chitosan is not clearly understood yet and its chemical nature is very similar to molecules generally used as chitosan crosslinkers. Therefore, these hydrogels are treated in this section.

The incorporation of an additional non-reacting polymer inside the network, such as gelatine, is possible and leads to the formation of a structure of the semi-IPN type, already presented in the first part. Crosslinking chitosan with sulfate or citrate can lead to precipitation. However, addition of gelatine, which should minimise interactions, followed by crosslinking at low temperature allows the formation of homogeneous gel beads [115].

Ionic crosslinking is a simple and mild procedure. In contrast to covalent crosslinking, no auxiliary molecules such as catalysts are required [107], which is of great interest for medical or pharmaceutical applications. Indeed, ionic crosslinking can be ensured by the classical method of preparing a crosslinked network, namely by the addition of the crosslinker, either solubilised [109] or dispersed [24,111], to the chitosan solution. These methods allow the formation of a homogeneous hydrogel by a random crosslinking reaction [111]. Other methods for ionic crosslinking of chitosan have also been developed to modulate hydrogel properties, such as drug release. Chitosan can be crosslinked by simply dipping pieces of chitosan film into the crosslinker solution [115] or by adding the chitosan solution to the crosslinker solution [106,108,116] through a syringe for example. These latter methods induce the formation of systems that are similar to

gel particles. These are formed by an unreacted core and a highly crosslinked surface that decreases and finally inhibits the diffusion of crosslinker towards the core of the network [108]. After crosslinking, networks can be further modified by coating with other polymers via PEC formation to prolong or modulate drug release. These polymers can be heparin, pectin [107] or alginate [107,116].

As in covalently crosslinked hydrogels, the crosslinking density is the main parameter influencing important properties of ionically crosslinked hydrogels, such as mechanical strength, swelling and drug release [117]. Therefore, it is important to determine the reaction conditions influencing the crosslinking density, in order to be able to modulate the properties of the network. The crosslinking reaction is mainly influenced by the size of the crosslinker and the global charges of chitosan and crosslinker during the reaction. The smaller the molecular size of the crosslinker, the faster is the crosslinking reaction, since its diffusion is easier [118]. With respect to the influence of the global charge, there is a difference between ions and ionic molecules. The charge density of ions depends on the oxidation number and is independent on the pH, whereas for ionic molecules, the global charge density depends on their pK_a values and on the pH of the solution during the reaction, as for chitosan [106,107,119], for which the macro pK_a is about 6.5 [120]. The global charge densities of chitosan and crosslinker must be sufficiently high in order to allow interactions and formation of a hydrogel. This means that the pH during the crosslinking reaction must be in the vicinity of the pK_a interval of the chitosan and the crosslinker. It should be noted that, if the pH is too high, the positive charges of chitosan are neutralised and the system is not ionically crosslinked but undergoes coacervation-phase inversion, since chitosan precipitates [118]. In order to avoid chitosan precipitation, the pH of the solution should not be higher than pH~6. This acidic pH can lower the biocompatibility of the system. The use of β-glycerophosphate allows neutralisation of the pH without chitosan precipitation, and therefore favours biocompatibility [121]. Particular attention should be paid to crosslinkers that can have a high charge density, ensuring a high crosslinking density, such as tripolyphosphate. Indeed, in order to allow a pH-dependent swelling with such crosslinkers, crosslinking should be incomplete [117]. This can be achieved by a short reaction time and a low crosslinker concentration [115]. Another possibility to obtain optimised networks, namely mechanically stable but with high swelling and drug release, is the combination of different crosslinkers, such as citrate and tripolyphosphate [115].

Besides the size of the crosslinker and the global charge densities, the crosslinking density is influenced by the addition of another polymer. Moreover, it is obviously influenced by the crosslinker concentration [24,115,122], as well as the MW [24], DD [109] and concentration of chitosan [24,113,122] and by the duration of the reaction [108,116,122].

Since the crosslinking density is an important parameter of the system, it is interesting to be able to follow the reaction during formation of the network. This can be performed with tripolyphosphate, which leads to the release of OH^- during the crosslinking reaction, allowing the reaction process to be monitored by pH measurement [106]. For the other hydrogels, the inability to follow the crosslinking reaction, renders the modulation of their properties more difficult. This aspect, therefore, warrants further investigation.

3.3. Properties and medical applications

Networks formed by ionic crosslinking of chitosan are mainly used for drug delivery. The properties of pH-dependent drug delivery systems can be controlled by the experimental conditions during preparation. They generally exhibit pH-sensitive swelling and drug release by diffusion [109,113,117] through their porous structure [109,111,123].

3.3.1. pH-sensitive swelling and drug release

Swelling is mainly influenced by ionic interactions between chitosan chains, which depend on the crosslinking density set during the formation of the network [106,108,113]. An increase in crosslinking density induces a decrease in swelling and pH-sensitivity, by improving the stability of the network [106,118], and results in decreased drug release. However, in ionically crosslinked hydrogels the crosslinking density is further modified by external conditions after administration, mainly by the pH of the application medium [106,107,124]. It influences the global charge densities of chitosan and crosslinker, which directly determine the crosslinking density, interactions and swelling. In contrast, in covalently crosslinked hydrogels, the crosslinking density is not modified after administration since these hydrogels are linked by irreversible bonds. Consequently, ionically crosslinked hydrogels cannot only swell in acidic but also in basic conditions (Fig. 3), which extends their potential applications. If the pH decreases, the charge density of the crosslinker and therefore the crosslinking density decrease, which leads to swelling. Moreover, swelling is favoured by the protonation and repulsion of chitosan free ammonium groups. If the pH decrease is too large, dissociation of ionic linkages and dissolution of the network can occur [107,118], leading to a fast drug release [115]. If the pH increases, the protonation of chitosan decreases and induces a decrease of the crosslinking density, allowing swelling. If the pH becomes too high, amino groups of chitosan are neutralised and ionic crosslinking is inhibited [111]. If the crosslinking density becomes too small, interactions are no longer strong enough to avoid dissolution and the ionic crosslinker is then released [108,111]. On the other hand, a covalently crosslinked hydrogel does not exhibit swelling in basic

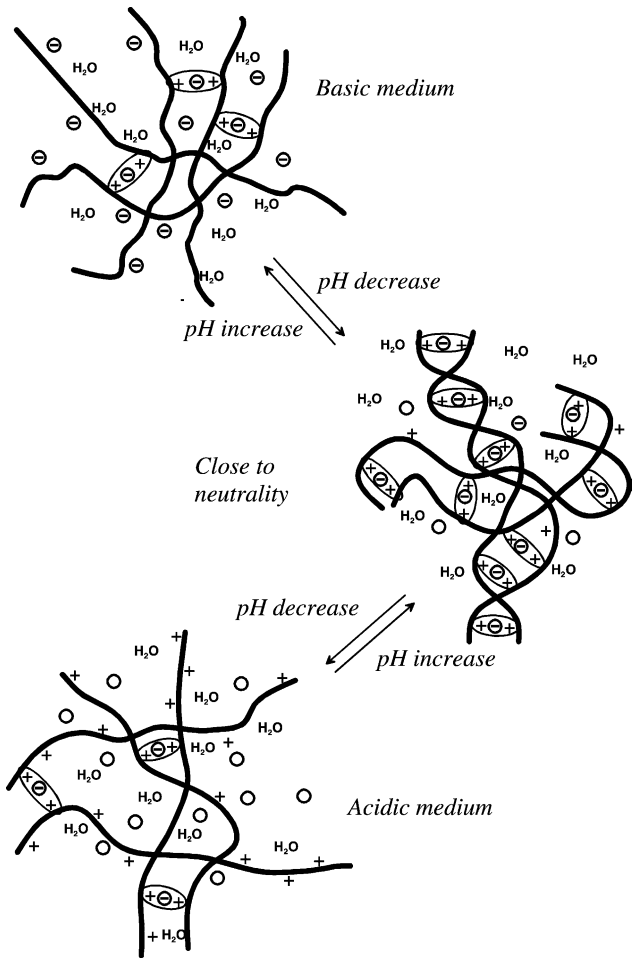

Fig. 3. pH-sensitive swelling of an ionically crosslinked chitosan hydrogel containing an ionic molecule as crosslinker; ⊖, charged ionic crosslinker; ○, uncharged ionic crosslinker; +, positive charge of chitosan; ⬭, ionic interaction; ▬, chitosan.

conditions and as the crosslinking density does not vary in a covalently crosslinked hydrogel, swelling is less pronounced but dissolution is avoided.

There are also some secondary parameters influencing swelling and release in ionically crosslinked hydrogels. In addition to its pH-sensitivity, swelling is ion-sensitive because the presence of ions weakens ionic interactions through a shielding effect, which increases swelling and delivery [107,111,115]. Moreover, a decrease of the chitosan MW lowers swelling and favours dissolution [106]. In addition, drug release depends on the solubility and MW of the drug [107] and is influenced by its concentration inside the network [110].

The additional formation of a semi-IPN after ionic crosslinking influences the drug delivery. Indeed, gelatine modifies drug release profiles and improves the mechanical strength of the network [115,116] and chondroitin sulfate [110] modulates porosity and release [110]. In addition, coating of networks via polyelectrolyte complexation strengthens the network and increases drug loading and sustained release capability [116].

3.3.2. Examples of use

Ionic crosslinking allows the formation of various systems exhibiting controlled drug delivery. Indeed, it is possible to prepare microspheres for the controlled release of 6-mercaptopurine [108], films for the controlled release of theophylline or 5-fluorouracil in the stomach [107] or sponges for the controlled release of platelet-derived growth factor-BB used in bone regeneration [110]. Other examples are given in Table 4. It should be noticed that the incorporation of active compounds such as guaifenesin, albumin or riboflavin certainly facilitates the measurement of release from these systems, but this is not the most interesting approach from a pharmacological point of view. Interestingly, the addition of β-glycerophosphate allows the preparation of a thermosetting hydrogel that can be easily injected and that favours bone regeneration [121]. Finally, the active compound in the matrix can also serve as the anion, as in the case of radioactive holmium-166. To the best of our knowledge, this example is the only chitosan hydrogel among those presented in this review, that has already been administered in humans during a clinical trial [6].

4. Advantages and disadvantages of crosslinked chitosan hydrogels

Among the four types of chitosan hydrogels presented in the introduction, covalently crosslinked hydrogels are the only systems characterised by a permanent network, due to their irreversible chemical links. Therefore, they exhibit good mechanical properties and can overcome dissolution, even in extreme pH conditions, while the other types of hydrogels are more labile. To obtain hydrogels with these interesting characteristics, the use of covalent crosslinkers is necessary. However, most of the crosslinkers used thus far are either known to be relatively toxic, or their fate in the human body is unknown and/or there is a lack of data concerning their biocompatibility. This requires to perform an additional purification and verification step before the administration of the hydrogel and may be problematic if free unreacted crosslinker is anyway found in traces in the hydrogel before administration. For the moment, the choice of safe, biocompatible covalent crosslinkers is quite limited, which is the main drawback of these systems. The use of derivatives of biocompatible polymers as crosslinkers represents a possible solution but their functionalisation often requires the use of auxiliary molecules, which have the same disadvantages as crosslinkers. Interesting alternatives are emerging, such as genipin. However, even with a safe biocompatible crosslinker, covalently crosslinked hydrogels would not necessarily be the best choice for

Table 4
Ionically crosslinked chitosan hydrogels

Type of system	Crosslinker	Additional polymer	Controlled release system	Biomedical applications
Chitosan–ion–chitosan	Ho-166	n.r.	Injectable hydrogel for radiation synovectomy by release of Ho-166 [6].	n.r.
Carboxymethylchitosan-ion-carboxymethylchitosan	Fe(III)	n.r.	Microspheres for the controlled release of 6-mercaptopurine [108].	n.r.
Chitosan-ionic molecule-chitosan	Calcium phosphate	n.r.	n.r.	Self hardening cement for use in dentistry [137].
	β-Glycerophosphate		Thermogelling hydrogel for the controlled release of chlorpheniramine or albumin [109]. Injectable thermogelling hydrogel for chondrocytes delivery [121].	n.r.
	Citrate		Film for controlled release of riboflavin, theophylline or 5- fluorouracile in the stomach [107].	n.r.
	Tripolyphosphate		Gel beads for controlled release of piroxicam [113] or 6- mercaptopurine [106]. Film for controlled delivery of chlorpheniramine maleate or guaifenesin [117]. Gel beads for release of lysine, bovine serum albumin or P-galactosidase [138].	Simulation of permeability of drugs through skin [122].
(Chitosan-ionic molecule-chitosan) + IPN	Tripolyphosphate	Chondroitin sulfate	Sponges for the controlled release of platelet-derived growth factor-BB used in bone regeneration [110].	n.r.
	Sulfate	Gelatine	Film for the improvement of drug sustained release performances [116].	n.r.
	Citrate		Gel beads for specific drug delivery in the stomach [115].	n.r.

n.r., no reference available.

each application since their use is limited by their lack of swelling and absence of pH-controlled release in basic conditions. Moreover, links can be disrupted when the hydrogel undergoes deformation, as during injection though a needle and a covalently crosslinked hydrogel does not spontaneously reform, unlike most physical hydrogels prepared by ionic crosslinking, complexation or aggregation. Covalently crosslinked chitosan hydrogels are interesting for applications where a well-shaped system is required, as for the formation of implants or bandages, or for the preparation of gel particles for oral administration exhibiting pH-dependent drug release in acidic conditions. Nevertheless, we believe that unless safe covalent crosslinkers with well-documented biocompatibility and metabolism are available, alternative hydrogels, such as ionically crosslinked hydrogels, should be preferred.

Ionic crosslinking is an extremely simple and mild method for preparing hydrogels. Moreover, ionically crosslinked chitosan hydrogels are generally thought to be well-tolerated and their potential medical and pharmaceutical applications are numerous since ionic crosslinkers are often biocompatible. Ionically crosslinked chitosan hydrogels offer more possibilities as drug delivery systems compared to covalently crosslinked hydrogels. They can be used for controlled release not only in acidic but also in basic media, for rapid release by dissolution and as thermogelling systems. However, their main disadvantages are the possible lack of mechanical stability and the risk of dissolution of the system, due to a highly pH-sensitive swelling.

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